

Sex-specific role for the 5-HT_{2A} receptor in psilocybin-induced extinction of opioid rewarding properties

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this manuscript, Jaster and colleagues have developed a model to investigate psilocybin's effects on two facets of repeated opioid exposure – place preference, and withdrawal symptoms. They found that that a single dose of psilocybin, after opioid exposure, reduces expressed place preference and somatic symptoms of withdrawal in male, but not female mice. These effects are 5HT_{2A}R dependent, some evidence is presented supporting a role for a frontal cortex->NAc circuit. The topic is timely and high impact, given the great interest in developing novel treatments for OUD and the relatively early stage research with psychedelics in this area. Most key experiments were well powered, appropriately analyzed and I am impressed to see such a serious effort to define sex dependent effects in mouse models- it should be done far more often. Overall, this paper would benefit from more focus – a considerable amount of data is presented, which undoubtedly represents a great amount of effort, but a sizeable chunk (eg Fig 7, 8, and a somewhat unfocused analysis of differential enhancers) contributes little to the overall claim of the paper. At the same time, one of the more interesting findings – the strong effect of psilocybin on opioid withdrawal behaviors – was not explored in the same depth as CPP, though it seems to me that an effect on opioid withdrawal would be a strong candidate to explain the reduction in OXY-CPP.

Major comments:

1. Results, para 1: Why did the authors choose the dose with maximal M/F difference? Was the plan to look for sex differences all along?
2. Fig 1c,d – what units is the half-life expressed in? 5.58 minutes does not match what I see on the graph, which appears to be closer to 30 minutes
3. Fig 1 e-l would benefit greatly from some sort of diagram to indicate whether the data represents acute or post-acute effects.
4. Fig 1g,h. Fig 2c,d. In general the authors have done a nice job of organizing the data and statistics – it could be even better if all of the statistical data were in one place rather than spread between captions and supplementary tables (the latter which is much easier to read).
5. Fig 1 i-l. Novel object. The text would be easier to understand if some brief description were given for “acquisition” and “recognition”, eg a simple diagram showing what is written in the methods. I am also having a difficult time understanding why “vehicle vs psi” gets a star in Fig 1L – none of the drug comparison terms are significant in Supp Table 3. There is a sex effect. It would be helpful to clarify in the text that psilocybin does not have sex dependent effects on any of these assays.
6. Fig 2a. Either in text or on the figure, it would be helpful to easily see the length of preference testing – a net preference score of ~100sec has a different meaning in a 10 vs 20 min testing session, for example.
7. Fig 2c,d. In general I have to applaud the authors for conducting well-powered studies and performing (and reporting) appropriate ANOVA tests for main effects and interactions. However, Fig 2c – the legend says “PSI reverses OXY-induced CPP”. The statistics show a significant interaction (presumably OXY x PSI, though that is not explicit in the caption, and the

Supp Table 4 shows results of a 3 way ANOVA including sex). The figure shows a star over the oxy – vs oxy + (psi -) comparison. Is that a posthoc test? I don't see post hoc test results listed in either the figure caption or Supp Table 4. I would reiterate my request to put all of the statistics in one place.

8. Fig 2 e-h. Here, the authors show that psilocybin, but not vehicle, uniquely reverses preference, and this effect only occurs in males but not females. I agree that the stats are consistent with the interpretation, but looking at the figures, the effect seems to derive entirely from a reduced variance in the Day 5 WT male / psilocybin group (panel f). I have a hard time convincing myself that the apparent reversal in panel f is not happening in females or in the vehicle group. I would be surprised if a two way ANOVA comparing within-mouse differences Day 6- Day 5, across four groups (veh vs psi x male vs female). This concern is less serious given the findings in the 5HT2aRKO (Fig 2l-o). It is pretty interesting – the trend across males and females suggests that 5ht2a is somehow important for extinction learning.

9. Fig 3 (withdrawal symptoms) is quite convincing, and adds weight to the idea that males are more sensitive to psilocybin than females in the context of opioids. Is it possible that the reduced withdrawal may also contribute to the reduced preference (seeking behavior) on day 6 in males? (fig 2f).

10. Fig 4c: rescue of 5HT2a in LSL mice. The rescue (left-most panel) is not particularly convincing- the “n.s.” may simply reflect an underpowered comparison – moreover, it seems that the attempt to restore 5HT2aR expression in FC-NAc did not really alter the phenotype observed in the 5HT2aR KO, (Fig 2m), further suggesting that perhaps the rescue of 5HT2aR expression was not very robust. I may be misunderstanding the graph, but it seems that the mean values for Day 6 on panel 4g and 4h should match the mean values showed on panel 4f, center two columns, which I understand to be measures taken on Day 6 as well. The fact that one of these comparisons (panel 4h) reached significance is not very convincing evidence that FC-NAc is driving their observed effect in Fig 1f. I would have been very interested to see whether this rescue strategy had any effect on psilocybin's attenuation of OXY withdrawal (Figs 3b-f), which was a much more robust effect than on CPP.

11. In the paragraph describing opioid receptor function in frontal cortex (Fig 5), authors conclude that the sex-dependent effect of psilocybin (apparently now showing up in females, not males) led them to explore epigenomic remodeling. I am unclear on the logic.

12. For the H3K27ac profiling (fig 6 and extended data fig 6), the text describes 4 groups (VEH-VEH, VEH-PSI, OXY-VEH, OXY-PSI), but the figure and caption only show 3 groups. Why is VEH-PSI left out?

13. “We further linked... based on Hi-C data”. For non-experts in this type of data, a brief explanation of Hi-C data would be very useful.

14. “our results suggest that PSI may counteract the effects of repeated OXY administration via cell-adhesion-associated pathways in male frontal cortex”. I don't follow the logic at all – looking at Supplemental Table 9, groups I, II and III all appear to have cell adhesion related genes at the top of each list, suggesting no particular effect of PSI. Was the authors' conclusion based on a quantitative finding? Further – the number of differential enhancers was much higher in NAc samples, so why focus on frontal cortex? Conclusions like “reactivation of genes linked to learning and memory may contribute to the observed reversal of opioid-induced CPP” is not appropriate in “results” without some supporting analysis or quantification – were learning and memory genes only observed in Group III? Additionally, there are not any groupings listed in “PFC F group 1” (Supplemental Table 9), though there are 73 genes noted in Fig 6d.

15. While interesting, spine analysis in NAc after PSI (Fig 8a-f) does not have any clear relationship with the previous data – it does not appear that these experiments were performed in opioid-exposed mice, so how do these data support any conclusions drawn from the foregoing data? The repeat PSI paradigm for CPP is also interesting, and it is honestly surprising that female mice express a preference at all. This finding may be worth following up, but I cannot see how effects observed after multiple doses of 2 mg/kg PSI over a 3 week period can inform conclusions about a single 1mg/kg exposure after OXY CPP (“PSI may not affect OXY-induced CPP in female mice due to its sex-specific confounding effects on rewarding behaviors”).

Minor

- Intro para 1: “agonists, such a methadone and buprenorphine”.. formally, buprenorphine is a partial agonist.
- Intro Para 2: “potentially transforming therapeutics” seems like a typo, “transformative”?
- Intro Para 2: Authors state psychedelics have been recently studied for SUDs including opioids – only studies for AUD and smoking cessation are cited.
- Results, para 1, sent 1: Awk phrasing “a mouse behavior signature of psychedelics upon activation”
- Fig 2b: Minor point – it's difficult to see the mean value on Fig 2a, please place mean / sem markers in the foreground
- “Sex differences in post-acute effects of PSI on OXY-induced CPP” : references to extended data should be updated to supplementary – I could only find Fig S1 S2 etc, no extended data.
- Fig 2e-h. Better labeling of the figures would (eg a legend) would help tremendously to keep track of the groups.
- Fig 3d, “%MPE” should be defined in the main text or caption
- Fig 4c, Main text refers to “other GPCR genes including 5-HT2cR (Htr2c)...”, though 5HT1aR is shown in panel 4c and described in the caption. Please either fix the graph or the text.
- It would help readers to define “GO” and “KEGG” in the main text.

(Remarks to the Author)

In the manuscript "Sex-specific role for the 5-HT2A receptor in psychedelic-induced extinction of opioid rewarding properties," Jaster and colleagues demonstrate some key distinctions in the neurobiological and behavioral response to psilocybin in male versus female mice, particularly with regards to its potential as a therapeutic for opioid use disorder (OUD). Serotonin 5-HT2A receptors located on prefrontal cortex pyramidal neurons projecting to the nucleus accumbens were found to be responsible for the therapeutic effect of psilocybin in males. This therapeutic effect was evident as facilitated extinction in a morphine conditioned place preference model in mice. Male and female mice also exhibited differential responsivity to acute psilocybin in terms of synaptic density in the nucleus accumbens, which was not dependent on the 5-HT2A receptor. Furthermore, the authors observed sex-specific epigenomic changes induced by psilocybin after chronic morphine exposure, with the male nucleus accumbens being more strongly impacted by psilocybin in terms of its ability to reverse opioid-induced pathology, and the frontal cortex of both sexes being responsive to psilocybin's effects on genes involved in morphogenesis, dendrite development, and synaptic function. The manuscript is a comprehensive dive into the sex differences of psilocybin as a therapeutic for OUD in terms of the underlying neurobiology. The experiments are well described, and the figures are appropriate. However, the manuscript could be improved by considering the following critiques and/or suggestions.

1. On p. 6 of the Results, in the last paragraph of the section titled "Sex differences in effect of PSI on unconditioned behaviors," it is stated that "female mice exhibited both a PSI effect (Fig. 1I), as well as a significant reduction in novel object exploration time compared to male animals (Fig. 1K, L, and Supplementary Table 3)." The directionality of this "PSI effect" (i.e. reduced or improved object recognition) is never explicitly mentioned in the main text here, but should be.
2. How do the authors reconcile the greater effect of DAMGO in the frontal cortex of females compared to males, when this is thought to be locus whereby psilocybin exerts its extinction-enhancing effects in males but not females?
3. The significance of the findings in Figure 8h and 8i could benefit from greater discussion as to why females would show a psilocybin-induced CPP and not males, and the relevance of this finding in the context of the sex differences in the underlying neurobiology shown in the remainder of the manuscript. If females are showing psilocybin rewarding effects and greater HTR at this same dose, is this why the dose is less therapeutic in females? Were higher doses tried in males, or lower doses in females?

Reviewer #3

(Remarks to the Author)

In this interesting and novel paper, the authors describe a sex-specific role for the 5-HT2A receptor in psilocybin-induced extinction of opioid rewarding properties. The paper is well written, the analyses performed are comprehensive and authors used classical and elegant strategies to decipher the role of the 5-HT2A receptor in opioid-seeking behavior and withdrawal symptoms. Overall, it is of great interest in the field of psychedelics and opioid effects. I have however some comments and suggestions that need to be the relevance of the manuscript

Major comments

- One major concern regards key conclusions that do not seem to be supported by the statistics. Authors interpret that the loss of statistical difference observed between the control group/Oxy treated group when animals are treated with PSI, as a reverting effect of PSI (Fig 2c, fig 3b). A similar error is made when restoring 5-HT2AR (Fig 4C) : the lack of statistical difference between Mock and the AAV-fDIO-Cre-eYFP does not implicate a restoration of the 5-HT2AR receptor. This is a critical issue in the manuscript that needs to be addressed.
- Concerning the results observed in females in the post-acute effects of PSI on OXY-induced CPP, we can observe 3 different subgroups in oxy-treated mice. Could authors elaborate on this? Recent data suggest that stage of estrous cycle significantly influenced oxycodone conditioned reward expression. Did authors check the estrous cycle in their cohorts?
- The data on MOR-G protein coupling are of interest. However, did the authors normalized on the quantity of receptors expressed? As increase in GTP binding could be related to differences in receptor expression
- The enrichment analysis study of the epigenomic data is broadly discussed, with a complicated presentation of the results. Moreover, all graphs are not presented, and authors only discuss selected Kegg pathways while omitting other when analyzing their results. This section of the manuscript and the data should be refined and clarified.

Minor comments

- The title is a bit vague cause it mentions "psychedelic-induced extinction of opioid rewarding properties". It exists several psychedelic molecules, such as lysergic acid diethylamide (LSD), psilocybin (PSI) and 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI). The authors only tested PSI in their experiments, so it would be more correct to specify this in the title (i.e. "psilocybin-induced extinction"). Otherwise, we might think that several psychedelics have been tested, which is not the case.
- The figures are somehow difficult to read, i.e. the information re: sex, genotype and other information is sometimes difficult to find

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Thank you, my concerns have been adequately addressed.

Reviewer #2

(Remarks to the Author)

The authors have made some suggested revisions to address my critiques. However, a few issues and/or areas to improve clarity remain, as outlined below.

1. Related to my previous comment #1, the authors added this verbiage to address the directionality of the PSI effect shown in females in Figure 1I: "However, female mice exhibited both a post-acute enhancement by PSI (Fig. 1I) and a significant reduction in novel object exploration time compared to male animals (Fig. 1k,l, and Supplementary Table 3)." This made me wonder when the PSI injection was administered during this task, and I could not find this information in the Figure legend, Methods, nor Results section of the manuscript. The use of the words "post-acute," implies the PSI was administered prior to acquisition. If so, how many minutes or hours prior? This is critical information for evaluating one of the two main behavioral tasks of the paper, yet I am struggling to find it mentioned once in the main text. It is clear that the PSI injection was administered 24-hour before the recognition test, but it is not clear the timing of the injection relative to acquisition, nor the timing of acquisition relative to recognition testing.

2. How do the authors reconcile the greater effect of DAMGO in the frontal cortex of females compared to males, when this is thought to be locus whereby psilocybin exerts its extinction-enhancing effects in males but not females?

The authors respond "There are two paragraphs in the Discussion focused on sex-related differences, with some edits based on Reviewers' comments." It is unclear which two paragraphs the authors are referring to that address this question. Most of the paragraphs in the discussion focus on sex-related differences. There is a paragraph on p. 20 (first full paragraph) that comes closest to addressing this question, which focuses on the methodological differences in assessing MOR density vs. DAMGO sensitivity, but this is not integrated with the question about the prefrontal cortex as a locus for PSI-enhanced extinction. It would be sufficient to address my question in the rebuttal, if the authors feel the Discussion is already too long, or this is beyond the scope of the Discussion, but I personally do not think that's the case.

3. The significance of the findings in Figure 8h and 8i could benefit from greater discussion as to why females would show a psilocybin-induced CPP and not males, and the relevance of this finding in the context of the sex differences in the underlying neurobiology shown in the remainder of the manuscript. If females are showing psilocybin rewarding effects and greater HTR at this same dose, is this why the dose is less therapeutic in females? Were higher doses tried in males, or lower doses in females?

The authors response "While the Reviewer's suggestion to test dose-response effects of repeated psilocybin administration on CPP – and eventually across all behavioral assays – is certainly of interest, the number of animals required to achieve sufficient statistical power in both sexes would exceed the scope of the current study," seems to have misinterpreted my question here. I was not asking to run a dose-response curve.

I am simply asking for an intellectual explanation that would reconcile the data presented in revised Fig 10h and i (old Fig 8h and i) compared to for example, Fig 2c and d. Is it possible that the rewarding effects of PSI are interfering with the OXY-reward memory differently between sexes? The Methods for CPP state that "Upon completion of testing all groups on day 5, mice were brought back to their home cages and given either PSI (1 mg/kg, i.p.) or vehicle." The dosing in the home cage should avoid interfering with any OXY-context representations, but the timing of the dosing with PSI could potentially interfere with reconsolidation of the OXY-CPP memory. At minimum, could the authors add information to the CPP Methods regarding the timing of the PSI/veh injections relative to removal from the CPP apparatus on day 5? If it was within 2 hours, this could disrupt reconsolidation. Was it a range of hours? At best, some Discussion of this seems to me to be highly relevant in the main text, unless I missed it.

Reviewer #3

(Remarks to the Author)

Thanks to the authors for addressing our comments, revised statistics and adding new results.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The authors addressed my concerns in their revisions.

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Reviewer #1 (Remarks to the Author):

In this manuscript, Jaster and colleagues have developed a model to investigate psilocybin's effects on two facets of repeated opioid exposure – place preference, and withdrawal symptoms. They found that that a single dose of psilocybin, after opioid exposure, reduces expressed place preference and somatic symptoms of withdrawal in male, but not female mice. These effects are 5HT_{2A}R dependent, some evidence is presented supporting a role for a frontal cortex->NAc circuit. The topic is timely and high impact, given the great interest in developing novel treatments for OUD and the relatively early stage research with psychedelics in this area. Most key experiments were well powered, appropriately analyzed and I am impressed to see such a serious effort to define sex dependent effects in mouse models- it should be done far more often. Overall, this paper would benefit from more focus – a considerable amount of data is presented, which undoubtedly represents a great amount of effort, but a sizeable chunk (eg Fig 7, 8, and a somewhat unfocused analysis of differential enhancers) contributes little to the overall claim of the paper. At the same time, one of the more interesting findings – the strong effect of psilocybin on opioid withdrawal behaviors – was not explored in the same depth as CPP, though it seems to me that an effect on opioid withdrawal would be a strong candidate to explain the reduction in OXY-CPP.

Major comments:

1. Results, para 1: Why did the authors choose the dose with maximal M/F difference? Was the plan to look for sex differences all along?

That's a perfectly valid concern. As mentioned in the first paragraph of the *Results* section (see also below), we selected this dose of psilocybin (1 mg/kg) for the remainder of our studies in this manuscript because it produced the most evident sex-related differences in the head-twitch response effect (a mouse proxy of human hallucinogenic potential).

"Since this difference was more evident at 1 mg/kg PSI (Fig. 1a and Extended Data Fig. 1a), and considering that pre-administration of the 5-HT_{2A}R antagonist volinanserine (0.1 mg/kg) fully blocked the effect of PSI (1 mg/kg) on HTR in both male and female mice (Fig. 1b and Extended Data Fig. 1b), we selected this dose of the psychedelic PSI for the remainder of studies."

2. Fig 1c,d – what units is the half-life expressed in? 5.58 minutes does not match what I see on the graph, which appears to be closer to 30 minutes

Half-life was initially calculated using the one-phase decay equation in GraphPad Prism 10. However, following Reviewer #1's comment, the same dataset has been reanalyzed in the Department of Pharmaceutics at VCU using the trapezoidal rule. This update is reflected in Fig 1c,d and the *Methods* section.

3. Fig 1 e-l would benefit greatly from some sort of diagram to indicate whether the data represents acute or post-acute effects.

A new diagram has been included in Fig S2a.

4. Fig 1g,h. Fig 2c,d. in general the authors have done a nice job of organizing the data and statistics – it could be even better if all of the statistical data were in one place rather than spread between captions and supplementary tables (the latter which is much easier to read).

The manuscript includes 14 supplementary tables, 10 of which present three-way ANOVA analyses. All Student's *t*-tests and two-way ANOVA results are described in the figure legends. In earliest drafts of this manuscript, we considered several formatting options, including one similar to the approach proposed by *Reviewer #1* (i.e., consolidating all three-way ANOVA analyses into a single supplementary table). However, due to the distinct experimental conditions associated with each dataset, combining them in one table made it more difficult to clearly associate each analysis with its corresponding figure or dataset. Therefore, while we appreciate the *Reviewer's* thoughtful suggestion, we believe that maintaining separate supplementary tables for each dataset improves clarity and readability of the manuscript.

5. Fig 1 i-l. Novel object. The text would be easier to understand if some brief description were given for “acquisition” and “recognition”, eg a simple diagram showing what is written in the methods. I am also having a difficult time understanding why “vehicle vs psi” gets a star in Fig 1L – none of the drug comparison terms are significant in Supp Table 3. There is a sex effect. It would be helpful to clarify in the text that psilocybin does not have sex dependent effects on any of these assays.

A new diagram illustrating the NOR test has been included in Fig S2b. Regarding data analysis of the NOR test, the most relevant information is presented in both the main text and figure legend. Using a two-way ANOVA (i.e., analyzing data from male and female mice separately), we observed a significant exploration effect (difference between acquisition and recognition) and also a psilocybin effect in the female group when testing exploration time (Fig 1I) (exploration $F[1,18] = 34.11$, $p < 0.001$; drug $F[1,18] = 5.45$, $p < 0.05$; interaction $F[1,18] = 0.21$, $p > 0.05$). This drug effect did not reach statistical significance in a three-way ANOVA (Table S3), likely because the other datasets (Figs. 1i–k) did not show similar effects in the two-way ANOVA analysis. Nevertheless, the three-way ANOVA revealed a clear sex effect on exploration time (Table S3), though not on exploratory preference (Table 2), as also shown in Figs. 1i–l. To improve clarity in presenting statistical results, we have revised the symbols: asterisks (*, **, ***) now denote *p*-values from post-hoc tests, while crosses (+, ++, ***) represent significant effects from two-way or three-way ANOVAs.

6. Fig 2a. Either in text or on the figure, it would be helpful to easily see the length of preference testing – a net preference score of ~100sec has a different meaning in a 10 vs 20 min testing session, for example.

The CPP method is described in detail in the *Methods* section. Additionally, following Reviewer's suggestion, we have now included the length of preference testing in the first paragraph of the *Results* section presenting CPP data.

"Male and female mice were habituated to CPP chambers (day 1), and then underwent OXY (3 mg/kg) or vehicle conditioning for three days (days 2-4). One day after the last conditioning session (day 5), animals were allowed to explore freely between the three compartments for 15 min, and time spent on each side was recorded (Fig. 2a)."

7. Fig 2c,d. In general I have to applaud the authors for conducting well-powered studies and performing (and reporting) appropriate ANOVA tests for main effects and interactions. However, Fig 2c – the legend says "PSI reverses OXY-induced CPP". The statistics show a significant interaction (presumably OXY x PSI, though that is not explicit in the caption, and the Supp Table 4 shows results of a 3 way ANOVA including sex). The figure shows a star over the oxy – vs oxy + (psi -) comparison. Is that a posthoc test? I don't see post hoc test results listed in either the figure caption or Supp Table 4. I would reiterate my request to put all of the statistics in one place.

We thank the Reviewer for this comment, which prompted us to revise how statistical comparisons are presented across all figure panels. As mentioned above, in the revised version of Fig 2c,d (and similar comparisons throughout the manuscript), we have added statistical symbols coming from two-way or three-way ANOVA analysis (+, **, ***) and from post-hoc tests (*, **, ***). For the post-hoc analysis, we also include all individual comparisons with the control group that did not reach statistical significance (n.s.). All relevant two-way ANOVA results (*i.e.*, main effects of OXY, PSI and their interaction in the case of Fig 2c,d) are shown in the corresponding figure legend.

8. Fig 2 e-h. Here, the authors show that psilocybin, but not vehicle, uniquely reverses preference, and this effect only occurs in males but not females. I agree that the stats are consistent with the interpretation, but looking at the figures, the effect seems to derive entirely from a reduced variance in the Day 5 WT male / psilocybin group (panel f). I have a hard time convincing myself that the apparent reversal in panel f is not happening in females or in the vehicle group. I would be surprised if a two way ANOVA comparing within-mouse differences Day 6- Day 5, across four groups (veh vs psi x male vs female). This concern is less serious given the findings in the 5HT2aRKO (Fig 2l-o). It is pretty interesting – the trend

across males and females suggests that 5ht2a is somehow important for extinction learning.

Because of the experimental design (shown in Fig 2a), we considered that paired Student's *t* test was the most appropriate statistical test to compare the effect of psilocybin vs vehicle in the same animals tested before and after administration. As mentioned by the *Reviewer*, the experiments are well powered and using this test, there was a statistically significant difference when comparing day 5 and day 6 in the WT male psilocybin group (Fig 2f), but not in the WT male vehicle group (Fig 2e). To support the sex-specific effect of these findings, our new data in the revised version of the manuscript show a similar phenotype induced by the phenethylamine psychedelic DOI (*i.e.*, DOI facilitates extinction of oxycodone preference in male but not in female mice – see Fig S5). Individual datapoints for the extinction learning effect are shown for each of the CPP experiments (Fig S3, Fig S6, Fig S8, Fig S11). It is important to mention that day 5 mice in Fig 2e and day 5 mice in Fig 2f belong to the same experimental group (*i.e.*, oxycodone-conditioned mice before vehicle or psilocybin administration). Because of this, and based on *Reviewer's* suggestion, we conducted a one-way ANOVA analysis comparing the three experimental groups in each of the CPP experiments (Fig S4, Fig S7, Fig S9, Fig S12). As an example (see Fig S4), this new analysis confirms (with a post-hoc analysis) that psilocybin (day 6), but not vehicle (day 6), is statistically different from the preference score on day 5 in male mice (but not in female mice). See also Fig S7, Fig S9 and Fig S12 for the same new statistical comparison.

9. Fig 3 (withdrawal symptoms) is quite convincing, and adds weight to the idea that males are more sensitive to psilocybin than females in the context of opioids. Is it possible that the reduced withdrawal may also contribute to the reduced preference (seeking behavior) on day 6 in males? (fig 2f).

Withdrawal symptoms would, in principle, be expected to reflect an aversion to the environment previously paired with oxycodone. However, this is not what we observed using the CPP model in our study. While psilocybin at the same dose (1 mg/kg) and tested at the same post-treatment time point (24-h after administration) affected both oxycodone-induced CPP and somatic withdrawal symptoms in male, but not female, mice, it is important to emphasize that the oxycodone administration protocols differed substantially between the two behavioral models. In the CPP paradigm, mice received oxycodone (3 mg/kg, s.c.) or vehicle once daily for three consecutive days during the conditioning period. In contrast, for assessing somatic withdrawal signs, we used a previously validated protocol (PMID: 38834159) designed to evaluate spontaneous withdrawal – without requiring a μ -opioid receptor antagonist such as naloxone. In this case, mice were implanted with subcutaneous osmotic minipumps delivering oxycodone at 60 mg/kg/day for seven days. These findings also showed that mice receiving subcutaneous (s.c.) injections of oxycodone (9–33 mg/kg, dose-response) twice daily for 8 days exhibited only mild spontaneous withdrawal symptoms. Given these differences in doses and route of administration, it would be premature to draw mechanistic

conclusions about a potential link between psilocybin's effects on CPP and withdrawal symptoms. Nevertheless, as noted by the *Reviewer*, our results clearly demonstrate that a single dose of psilocybin (1 mg/kg) post-acutely (24-h later) reduced both oxycodone-induced CPP and somatic withdrawal signs in male but not female mice. Furthermore, this sex-specific effect in males was 5-HT_{2A}R-dependent, as it was absent in 5-HT_{2A}R-KO mice.

10. Fig 4c: rescue of 5HT2a in LSL mice. The rescue (left-most panel) is not particularly convincing- the “n.s.” may simply reflect an underpowered comparison – moreover, it seems that the attempt to restore 5HT2aR expression in FC-NAc did not really alter the phenotype observed in the 5HT2aR KO, (Fig 2m), further suggesting that perhaps the rescue of 5HT2aR expression was not very robust. I may be misunderstanding the graph, but it seems that the mean values for Day 6 on panel 4g and 4h should match the mean values showed on panel 4f, center two columns, which I understand to be measures taken on Day 6 as well. The fact that one of these comparisons (panel 4h) reached significance is not very convincing evidence that FC-NAc is driving their observed effect in Fig 1f. I would have been very interested to see whether this rescue strategy had any effect on psilocybin's attenuation of OXY withdrawal (Figs 3b-f), which was a much more robust effect than on CPP.

Regarding the rescue of 5-HT_{2A}R expression in LSL mice, we have i) increased the number of replicates (new AAV/AAVr surgeries) and conducted a new ANOVA followed by post-hoc. The results show that both WT (internal control) and AAV/AAVr LSL mice exhibit significantly higher 5-HT_{2A}R mRNA expression in the frontal cortex compared to the KO group (Fig. 5d). This finding is further supported by new immunoblot assays using an anti-5-HT_{2A}R antibody in frontal cortex samples from WT, KO, and AAV/AAVr LSL mice (Fig. 5c and Fig. S10).

Regarding the data in Figs. 6c, 6d, and 6e (which were part of Fig. 4 in the previous version of the manuscript), Fig. 6c shows preference on day 6 (“post-conditioning score”), calculated by subtracting the time (in seconds) spent on the saline-paired side from the time spent on the oxycodone-paired side during the test. In contrast, Figs. 6d and 6e present the “preference score” as a measure of extinction learning across days 5 and 6. This within-subject measure was determined by subtracting the time (in seconds) spent on the drug-paired side on the habituation day from that on days 5 and 6, respectively. These analyses follow established methods used in our lab and by others in the field (PMID: 35183539; PMID: 37316665). Importantly, our current data demonstrate that these two behavioral pharmacology phenotypes (*i.e.*, expression of preference, and extinction learning) can be differentially affected depending on the psychedelic. For instance, psilocybin reduces the expression of oxycodone preference (Fig. 2c,d) and facilitates extinction learning (Fig. 2e-h), but only in male mice. Interestingly, our new data show that 24-h after DOI administration, neither male nor female mice displayed a significant reduction in the expression of oxycodone preference (Fig. S5b,c). However,

the same cohort of male mice conditioned with oxycodone and subsequently treated with DOI (but not saline) exhibited a significant reduction in preference between day 5 (pre-DOI) and day 6 (24-h post-treatment) (Fig. S5d-g). This suggests that DOI does not affect the expression of oxycodone preference per se but may selectively facilitate extinction learning. Related to molecular mechanisms, our data show the post-acute effects of psilocybin on both preference score and extinction learning observed in male mice are absent in *5-HT_{2A}R-KO* mice. Related to circuit, our data (as mentioned above) clearly show that the AAV/AAVr approach restores 5-HT_{2A}R expression in frontal cortex pyramidal neurons projecting to the NAc of otherwise full KO mice. Additionally, our data show that 5-HT_{2A}R within this particular circuit is sufficient to induce the psilocybin-dependent effects on facilitation of extinction learning, but not on expression of oxycodone preference. This is further supported by a one-way ANOVA analysis of the extinction learning dataset in *LSL* mice (Fig. S12).

As discussed above, most of our efforts in this paper are focused on the rewarding aspects of the opioid drug oxycodone. However, the data on somatic symptoms of opioid withdrawal further support the potential translational utility of psilocybin for individuals with OUD. These findings also suggest sex differences (effects observed in male but not female mice) and implicate 5-HT_{2A}R-dependent mechanisms. As noted earlier, the behavioral pharmacology methods used in the CPP and somatic withdrawal models (e.g., opioid dose, route of administration, and time course) are not directly comparable. Therefore, at the current stage of the research, we believe it would be overambitious to delineate the specific brain circuits underlying the 5-HT_{2A}R-dependent and sex-specific effects of psilocybin on oxycodone withdrawal. Given the novelty and significance of the findings already presented in the current version of the manuscript, we hope *Reviewer #1* agrees that a follow-up study – focusing specifically on the neural circuits involved in different models of opioid withdrawal – would be a more appropriate venue for those data.

11. In the paragraph describing opioid receptor function in frontal cortex (Fig 5), authors conclude that the sex-dependent effect of psilocybin (apparently now showing up in females, not males) led them to explore epigenomic remodeling. I am unclear on the logic.

New data in the revised version of the manuscript indicate that a single administration of psilocybin selectively attenuates MOR-mediated G protein coupling and reduces MOR mRNA expression in the frontal cortex of female mice, without altering overall MOR protein levels, suggesting sex-specific modulation of MOR signaling that is not observed in the NAc (Fig. 7).

Additionally, we have rephrased the first sentence of the *Results* describing the epigenomic assays, and added a new paragraph to the *Discussion* section.

“Building on these sex-specific effects at the MOR signaling level, we next assessed whether PSI alters the epigenomic landscape in a sex-dependent manner following repeated opioid exposure.”

“It remains to be determined how a single administration of PSI leads to reduced MOR mRNA expression and attenuated G protein coupling in the frontal cortex in a sex-specific manner, despite no detectable changes in total MOR density as assessed by [³H]naloxone binding assays following post-acute PSI treatment. It is worth noting, however, that [³H]naloxone binding was conducted using total membrane preparations from crude homogenates, whereas DAMGO-stimulated [³⁵S]GTPγS binding assays were performed on synaptosomal fractions (i.e., P2 membranes). This methodological difference may suggest that, at 24-h post-PSI administration, MOR trafficking or subcellular localization – and consequently, the ability of MOR to recruit heterotrimeric G proteins – is altered, even total receptor levels remain unchanged.”

12. For the H3K27ac profiling (fig 6 and extended data fig 6), the text describes 4 groups (VEH-VEH, VEH-PSI, OXY-VEH, OXY-PSI), but the figure and caption only show 3 groups. Why is VEH-PSI left out?

The Reviewer raised a very reasonable question. VEH-PSI is actually one of the groups included in Figs 8a,b (blue bars) (which were part of Fig. 6 in the previous version of the manuscript). In these two panels, when VEH-PSI is compared to VEH-VEH (control), our results indicate how PSI treatment alone affects this epigenomic profile in the frontal cortex and NAc. However, the overarching goal of the epigenomic analysis in this study is to examine how psilocybin treatment potentially affects the alterations induced after repeated oxycodone administration. Thus, we later mostly focus on the comparison between OXY-VEH and OXY-PSI. This comparison directly illustrates the impact of psilocybin administration on oxycodone-treated male and female mice. This rationale is included as part of the *Results* section:

“In order to decipher the epigenomic changes associated with repeated opioid exposure, both alone and in combination with psychedelic administration, we divided the differential enhancer-linked genes associated with OXY-VEH and OXY-PSI into three groups (Fig. 8c-f): i) genes altered exclusively in the OXY-VEH group (these genes are altered by repeated OXY administration but recover to the reference state after post-acute PSI treatment); ii) overlapping genes (these genes are altered by repeated OXY administration and remain altered after post-acute PSI treatment); and iii) genes exclusively altered in the OXY-PSI group.”

13. “We further linked... based on Hi-C data”. For non-experts in this type of data, a brief explanation of Hi-C data would be very useful.

We have added a brief explanation of Hi-C method and how it is applied in our analysis:

“To ensure high accuracy gene annotation, differential enhancers were overlapped with previously published datasets generated using the Hi-C method, a technique which evaluates chromatin conformation based on frequency of DNA fragment physical associations⁴². Hi-C data allow identification of long-range contacts between enhancer and genes⁴³.”

14. “our results suggest that PSI may counteract the effects of repeated OXY administration via cell-adhesion-associated pathways in male frontal cortex”. I don’t follow the logic at all – looking at Supplemental Table 9, groups I, II and III all appear to have cell adhesion related genes at the top of each list, suggesting no particular effect of PSI. Was the authors’ conclusion based on a quantitative

finding? Further – the number of differential enhancers was much higher in NAc samples, so why focus on frontal cortex? Conclusions like “reactivation of genes linked to learning and memory may contribute to the observed reversal of opioid-induced CPP” is not appropriate in “results” without some supporting analysis or quantification – were learning and memory genes only observed in Group III? Additionally, there are not any groupings listed in “PFC F group 1” (Supplemental Table 9), though there are 73 genes noted in Fig 6d.

Upon further examination, although the “cell-substrate adhesion” terms are fairly uniquely in Group I, “cell junction assembly” terms are highly ranked in all three groups. Cell junctions are closely related to cell-substrate adhesion. Thus, we agree with the Reviewer that our previous statement may be problematic. We have removed this part from the manuscript.

All experiments/analyses performed with frontal cortex tissue samples were also carried out on the NAc. The two brain regions were discussed in about equal portions. Some epigenomic features may be more pronounced in one region than the other.

In order to clarify our mention of sex difference enrichment in “learning & memory” related GO terms, the following sentence has been added to the manuscript:

“Similar learning and memory related terms were notably absent for the female mice.”

The 73 genes in Fig. 7d (which was Fig 6d in the previous version of the manuscript) did not yield any statistically significant KEGG terms. This is also the case for a number of other groups of genes analyzed. We added to the legend of Fig 7:

“The complete lists of terms are included in Supplementary Table 9. Only groups that yield enriched terms are included.”

15. While interesting, spine analysis in NAc after PSI (Fig 8a-f) does not have any clear relationship with the previous data – it does not appear that these experiments were performed in opioid-exposed mice, so how do these data support any conclusions drawn from the foregoing data? The repeat PSI paradigm for CPP is also interesting, and it is honestly surprising that female mice express a preference at all. This finding may be worth following up, but I cannot see how effects observed after multiple doses of 2 mg/kg PSI over a 3 week period can inform conclusions about a single 1mg/kg exposure after OXY CPP (“PSI may not affect OXY-induced CPP in female mice due to its sex-specific confounding effects on rewarding behaviors”).

We thank the Reviewer for this comment. The number of clinical studies investigating the potentially therapeutic effects of classical psychedelics such as psilocybin is rapidly increasing. In preclinical models, most studies on the synaptic and structural plasticity effects of psychedelics have focused on the frontal cortex (PMID: 34686347; PMID: 29898390; PMID: 34228959; PMID: 40175553). However, as noted in our manuscript, previous work by other groups clearly showed that repeated administration of drugs with high abuse liability, such as cocaine and morphine, induces dendritic structural plasticity

changes in subcortical brain regions involved in reward processing, including the NAc. To our knowledge, our study is the first to report sex-specific effects of a single dose of psilocybin (*i.e.*, administered in an approach consistent with clinical protocols) on dendritic spine density in the NAc. These findings, along with our data showing sex-specific effects of psilocybin on oxycodone-induced CPP and withdrawal, underscore the importance of considering sex as a biological variable in studies of psychedelic-assisted treatments. We believe these results will be of significant interest to both preclinical and clinical researchers working with psychedelics, as well as those investigating potential sex differences and sex bias in drug abuse liability. We also emphasize that the dendritic structural plasticity changes were observed following a single administration of psilocybin vs vehicle, consistent with the paradigm used in our study, which combines repeated oxycodone exposure with a single psychedelic dose. Nonetheless, we appreciate the *Reviewer's* comment regarding effect of repeated psilocybin administration on CPP, and we have revised the *Discussion* accordingly.

Minor

- Intro para 1: “agonists, such a methadone and buprenorphine”.. formally, buprenorphine is a partial agonist.

Buprenorphine is now defined as a partial agonist.

- Intro Para 2: “potentially transforming therapeutics” seems like a typo, “transformative”?

This typo has been corrected.

- Intro Para 2: Authors state psychedelics have been recently studied for SUDs including opioids – only studies for AUD and smoking cessation are cited.

A new reference has been added:

“In the past decade, psychedelics have been studied as potentially transformative therapeutics for a variety of neuropsychiatric disorders such as depression and substance use disorders (SUDs) including tobacco and alcohol^{11,12}, and most recently, opioids¹³.”

- Results, para 1, sent 1: Awk phrasing “a mouse behavior signature of psychedelics upon activation”

This sentence has been edited:

“To explore the potential role of 5-HT_{2A}R in the post-acute effects of PSI on OUD models, we first tested its effect on head-twitch behavior (HTR), a mouse behavioral proxy for human psychedelic potential^{17,28}.”

- Fig 2b: Minor point – it's difficult to see the mean value on Fig 2a, please place mean / sem markers in the foreground

Fig 2b, Fig 2i, and Fig S5a have been edited, following Reviewer's suggestion.

- "Sex differences in post-acute effects of PSI on OXY-induced CPP" : references to extended data should be updated to supplementary – I could only find Fig S1 S2 etc, no extended data.

All Extended Data Figs. are now properly referenced in the *Results* section.

- Fig 2e-h. Better labeling of the figures would (eg a legend) would help tremendously to keep track of the groups.

There is a timeline in Fig 2a, a brief description of the timepoints in Fig 2e-h's legend, and a full description of the CPP paradigm in the *Methods* section.

- Fig 3d, "%MPE" should be defined in the main text or caption

This abbreviation had previously been defined in the *Methods* section, and is now included in the legend of Fig. 3.

- Fig 4c, Main text refers to "other GPCR genes including 5-HT2cR (Htr2c)...", though 5HT1aR is shown in panel 4c and described in the caption. Please either fix the graph or the text.

This typo has been corrected

- It would help readers to define "GO" and "KEGG" in the main text.

These two abbreviations have been expanded.

Reviewer #2 (Remarks to the Author):

In the manuscript "Sex-specific role for the 5-HT2A receptor in psychedelic-induced extinction of opioid rewarding properties," Jaster and colleagues demonstrate some key distinctions in the neurobiological and behavioral response to psilocybin in male versus female mice, particularly with regards to its potential as a therapeutic for opioid use disorder (OUD). Serotonin 5-HT2A receptors located on prefrontal cortex pyramidal neurons projecting to the nucleus accumbens were found to be responsible for the therapeutic effect of psilocybin in males. This therapeutic effect was evident as facilitated extinction in

a morphine conditioned place preference model in mice. Male and female mice also exhibited differential responsivity to acute psilocybin in terms of synaptic density in the nucleus accumbens, which was not dependent on the 5-HT2A receptor. Furthermore, the authors observed sex-specific epigenomic changes induced by psilocybin after chronic morphine exposure, with the male nucleus accumbens being more strongly impacted by psilocybin in terms of its ability to reverse opioid-induced pathology, and the frontal cortex of both sexes being responsive to psilocybin's effects on genes involved in morphogenesis, dendrite development, and synaptic function. The manuscript is a comprehensive dive into the sex differences of psilocybin as a therapeutic for OUD in terms of the underlying neurobiology. The experiments are well described, and the figures are appropriate. However, the manuscript could be improved by considering the following critiques and/or suggestions.

1. On p. 6 of the Results, in the last paragraph of the section titled "Sex differences in effect of PSI on unconditioned behaviors," it is stated that "female mice exhibited both a PSI effect (Fig. 1I), as well as a significant reduction in novel object exploration time compared to male animals (Fig. 1k,l, and Supplementary Table 3)." The directionality of this "PSI effect" (i.e. reduced or improved object recognition) is never explicitly mentioned in the main text here, but should be.

This perfectly valid minor concern has been addressed in both the main text and in the Fig 1I, showing the statistical significance of the post-hoc test comparing acquisition/vehicle vs acquisition/psilocybin for exploration time in the female group.

"However, female mice exhibited both a post-acute enhancement by PSI (Fig. 1I) and a significant reduction in novel object exploration time compared to male animals (Fig. 1k,l, and Supplementary Table 3)."

2. How do the authors reconcile the greater effect of DAMGO in the frontal cortex of females compared to males, when this is thought to be locus whereby psilocybin exerts its extinction-enhancing effects in males but not females?

There are two paragraphs in the *Discussion* focused on sex-related differences, with some edits based on *Reviewers'* comments.

3. The significance of the findings in Figure 8h and 8i could benefit from greater discussion as to why females would show a psilocybin-induced CPP and not males, and the relevance of this finding in the context of the sex differences in the underlying neurobiology shown in the remainder of the manuscript. If females are showing psilocybin rewarding effects and greater HTR at this same dose, is this why the dose is less therapeutic in females? Were higher doses tried in males, or lower doses in females?

An additional sentence highlighting potential mechanisms underlying these sex differences in psilocybin-induced CPP has been added to the *Discussion* section (see below). As mentioned in the first paragraph of the *Results* section, we selected a dose of 1 mg/kg psilocybin for the remainder of our studies because it produced the most evident sex-related differences in the head-twitch response, a widely used mouse proxy for the hallucinogenic potential of psychedelics in humans. To our knowledge, this is the first report demonstrating sex-specific effects of classical psychedelics (including psilocybin and DOI) on translationally relevant post-acute effects in rodent models of opioid use disorder. While the *Reviewer's* suggestion to test dose-response effects of repeated psilocybin administration on CPP – and eventually across all behavioral assays – is certainly of interest, the number of animals required to achieve sufficient statistical power in both sexes would exceed the scope of the current study. Nevertheless, we have acknowledged this limitation and included it as a potential direction for future investigation in the *Discussion* section (see below).

"Similarly, further investigation will examine interactions with the estrous cycle and the role of ovarian hormones in regulating psychedelic-induced plasticity in rodents^{78,79}."

"Future studies using a dose-response approach to evaluate the post-acute effects of PSI administration in both sexes will be valuable to fully capture the range of behavioral and neurobiological adaptations that may underlie these sex-specific outcomes."

Reviewer #3 (Remarks to the Author):

In this interesting and novel paper, the authors describe a sex-specific role for the 5-HT2A receptor in psilocybin-induced extinction of opioid rewarding properties. The paper is well written, the analyses performed are comprehensive and authors used classical and elegant strategies to decipher the role of the 5-HT2A receptor in opioid-seeking behavior and withdrawal symptoms. Overall, it is of great interest in the field of psychedelics and opioid effects. I have however some comments and suggestions that need to be the relevance of the manuscript

Major comments

• One major concern regards key conclusions that do not seem to be supported by the statistics. Authors interpret that the loss of statistical difference observed between the control group/Oxy treated group when animals are treated with PSI, as a reverting effect of PSI (Fig 2c, fig 3b). A similar error is made when restoring 5-HT2AR (Fig 4C) : the lack of statistical difference between Mock and the AAV-fDIO-Cre-eYFP does not implicate a restoration of the 5-HT2AR receptor. This is a critical issue in the manuscript that needs to be addressed.

Regarding Fig 2c, we conducted a two-way ANOVA analysis evaluating effects of psilocybin, repeated oxycodone, and interaction. We show (see Fig 2c's legend) that there was a statistically significant interaction effect ($p < 0.01$) between psilocybin and repeated oxycodone. Following this, a post-hoc analysis comparing the vehicle control condition against the oxycodone alone, oxycodone and psilocybin, and psilocybin alone

conditions shows a statistically difference between the vehicle control group and oxycodone alone, but not between the vehicle control group and other two groups (oxycodone and psilocybin, and psilocybin alone). See also Table S4 for a three-way ANOVA analysis.

Additionally, to improve clarity in presenting statistical results (and based on *Reviewer's* comment regarding the reverting effect of psilocybin), we have revised the symbols: asterisks (*, **, ***) now denote p-values from post-hoc tests, while crosses (+, ++, ***) represent significant effects from two-way or three-way ANOVAs throughout.

Regarding Fig 3b (and similarly for the current Figs 3c-e), our statistical analysis in the previous version of the manuscript was based on a two-way ANOVA analysis with drug treatment (oxycodone and/or psilocybin) and sex as the two independent variables. Based on *Reviewer's* perfectly valid suggestion, we conducted a new analysis using the same datasets, applying a statistical approach similar to that used for the CPP datasets (e.g., Fig 2c,d). Specifically, we performed a two-way ANOVA to evaluate the effects of drug administration (oxycodone and psilocybin) as two independent experimental factors. This analysis revealed significant main effects of oxycodone ($p < 0.001$), psilocybin ($p < 0.001$), and a significant interaction between them ($p < 0.001$) in wild-type male mice (Fig. 3a). In contrast, only a significant oxycodone effect was observed in wild-type female, KO male, and KO female mice (Figs. 3c-e). In addition, sex and genotype effects were evaluated using a three-way ANOVA (see Supplementary Table 5). A similar change in the data analysis has been include in Figs. 3g,i (tail-flick test).

Regarding Fig 5d (which was Fig 4c in the previous version of the manuscript), we have i) increased the number of replicates (new AAV/AAVr surgeries) and ii) conducted a new ANOVA followed by post-hoc. The results show that both WT (internal control) and AAV/AAVr LSL mice exhibit significantly higher 5-HT_{2A}R mRNA expression in the frontal cortex compared to the KO group (Fig. 5d). This finding is further supported by new immunoblot assays using an anti-5-HT_{2A}R antibody in frontal cortex samples from WT, KO, and AAV/AAVr LSL mice (Fig. 5d and Fig. S10).

• Concerning the results observed in females in the post-acute effects of PSI on OXY-induced CPP, we can observe 3 different subgroups in oxy-treated mice. Could authors elaborate on this? Recent data suggest that stage of estrous cycle significantly influenced oxycodone conditioned reward expression. Did authors check the estrous cycle in their cohorts?

We run several statistical tests for normal distribution of the OXY and OXY-PSI groups in Fig 2d, and they all passed the normality test. This potential “3 different sub-groups” in OXY-treated female mice has not been observed in a separate cohort of female mice tested with oxycodone and the psychedelic DOI (see Fig S5c). However, given the sex-related differences observed in this manuscript, and recent findings by other groups indicating estrous-dependent effect of psilocybin-induced head-twitch behavior in rats

(doi: <https://doi.org/10.1101/2025.01.10.632408>), this has been mentioned in the *Discussion* as an interesting follow-up work.

“Similarly, further investigation will examine interactions with the estrous cycle and the role of ovarian hormones in regulating psychedelic-induced plasticity in rodents^{78,79}. “

• The data on MOR-G protein coupling are of interest. However, did the authors normalized on the quantity of receptors expressed? As increase in GTP binding could be related to differences in receptor expression

Following Reviewer’s comments, we have conducted additional experiments in separate cohorts of mice testing MOR mRNA (RT-qPCR) and MOR protein ([³H]naloxone binding) in the frontal cortex of male and female mice – samples were collected 24-h after a single dose of psilocybin, or vehicle (Figs. 7c-e).

• The enrichment analysis study of the epigenomic data is broadly discussed, with a complicated presentation of the results. Moreover, all graphs are not presented, and authors only discuss selected Kegg pathways while omitting other when analyzing their results. This section of the manuscript and the data should be refined and clarified.

All the groups of genes underwent GO and KEGG enrichment analyses. Some of these groups do not yield any enriched terms. All the groups of genes that yield any enriched KEGG terms were included in Fig. 8 (which was Fig 6 in the previous version of the manuscript). To clarify, we added in Fig. 7’s legend:

“The complete lists of terms are included in Supplementary Table 9. Only groups that yield enriched terms are included”

We also added the following definitions for GO and KEGG on pages 12-13 along with citations for improved clarity:

“Next, we conducted GO analysis – a ranked statistical approach used to identify enriched gene clusters based on shared functional characteristics associated with specific biological processes^{45,46} – across various groups of genes”.

“KEGG pathway analysis – a method that draws upon an index of biological pathways and networks associated with enriched gene clusters⁴⁹ – was conducted for Group I, Group II and Group III genes in the frontal cortex (Fig. 8g-i) and NAc (Fig. 8j-o) of both sexes.”

For KEGG and GO analysis, terms are counted and statistically analyzed to identify enriched terms compared to the background. We then analyze significantly enriched (FDR < 0.05) terms based on existing knowledge of the literature to highlight those with

established links to substance abuse. The full lists of genes, GO/KEGG terms are included in Supplementary Table 9 for readers' reference.

Minor comments

• ***The title is a bit vague cause it mentions “psychedelic-induced extinction of opioid rewarding properties”. It exists several psychedelic molecules, such as lysergic acid diethylamide (LSD), psilocybin (PSI) and 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI). The authors only tested PSI in their experiments, so it would be more correct to specify this in the title (i.e. “psilocybin-induced extinction”). Otherwise, we might think that several psychedelics have been tested, which is not the case.***

New data indicate that DOI also affects oxycodone-induced CPP in a sex-dependent manner (Fig. S5). That said, we are following Reviewer #3's suggestion and the title has been changed to: “Sex-specific role for the 5-HT_{2A} receptor in psilocybin-induced extinction of opioid rewarding properties”

• ***The figures are somehow difficult to read, i.e. the information re: sex, genotype and other information is sometimes difficult to find***

Figures and figure legends have been edited including additional statistical comparisons among groups.

Reviewer #4 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #2 (Remarks to the Author):

The authors have made some suggested revisions to address my critiques. However, a few issues and/or areas to improve clarity remain, as outlined below.

1. Related to my previous comment #1, the authors added this verbiage to address the directionality of the PSI effect shown in females in Figure 1I: “However, female mice exhibited both a post-acute enhancement by PSI (Fig. 1I) and a significant reduction in novel object exploration time compared to male animals (Fig. 1k,l, and Supplementary Table 3).” This made me wonder when the PSI injection was administered during this task, and I could not find this information in the Figure legend, Methods, nor Results section of the manuscript. The use of the words “post-acute,” implies the PSI was administered prior to acquisition. If so, how many minutes or hours prior? This is critical information for evaluating one of the two main behavioral tasks of the paper, yet I am struggling to find it mentioned once in the main text. It is clear that the PSI injection was administered 24-hour before the recognition test, but it is not clear the timing of the injection relative to acquisition, nor the timing of acquisition relative to recognition testing.

Additional details related to the novel object recognition protocol are included in the *Methods* section and in the legend of Fig S2.

Methods section:

Novel object recognition – assessed as preference for a novel object over a familiar one – was tested 24-h after mice received either PSI (1 mg/kg, i.p.) or vehicle (Extended Data Fig. 2b).

Fig. S2 (legend):

b, Novel object recognition – assessed as preference for a novel object over a familiar one – was tested 24-h after mice received either PSI (1 mg/kg, i.p.) or vehicle.

2. How do the authors reconcile the greater effect of DAMGO in the frontal cortex of females compared to males, when this is thought to be locus whereby psilocybin exerts its extinction-enhancing effects in males but not females?

The authors respond “There are two paragraphs in the Discussion focused on sex-related differences, with some edits based on Reviewers’ comments.” It is unclear which two paragraphs the authors are referring to that address this question. Most of the paragraphs in the discussion focus on sex-related differences. There is a paragraph on p. 20 (first full paragraph) that comes closest to addressing this question, which focuses on the methodological differences in assessing MOR density vs. DAMGO sensitivity, but this is not integrated with the question about the prefrontal cortex as a locus for PSI-enhanced extinction. It would be sufficient to address my question in the rebuttal, if the authors feel the Discussion is already too long, or this is beyond the scope of the Discussion, but I personally do not think that’s the case.

An additional sentence has been added to the corresponding paragraph in the *Discussion* section.

Differences in GPCR binding and functional outcomes may contribute to sex differences in both 5-HT_{2A}R- and MOR-dependent effects, potentially due to pharmacological crosstalk between the two receptors⁶⁷. Previous^{28,30} and current data indicate that 5-HT_{2A}R density is decreased, while both MOR density and DAMGO-induced MOR-G protein coupling are increased in the frontal cortex of female mice compared to males.

3. The significance of the findings in Figure 8h and 8i could benefit from greater discussion as to why females would show a psilocybin-induced CPP and not males, and the relevance of this finding in the context of the sex differences in the underlying neurobiology shown in the remainder of the manuscript. If females are showing psilocybin rewarding effects and greater HTR at this same dose, is this why the dose is less therapeutic in females? Were higher doses tried in males, or lower doses in females?

The authors response “While the Reviewer’s suggestion to test dose-response effects of repeated psilocybin administration on CPP – and eventually across all behavioral assays – is certainly of interest, the number of animals required to achieve sufficient statistical power in both sexes would exceed the scope of the current study,” seems to have misinterpreted my question here. I was not asking to run a dose-response curve.

I am simply asking for an intellectual explanation that would reconcile the data presented in revised Fig 10h and i (old Fig 8h and i) compared to for example, Fig 2c and d. Is it possible that the rewarding effects of PSI are interfering with the OXY-reward memory differently between sexes? The Methods for CPP state that “Upon completion of testing all groups on day 5, mice were brought back to their home cages and given either PSI (1mg/kg, i.p.) or vehicle.” The dosing in the home cage should avoid interfering with any OXY-context representations, but the timing of the dosing with PSI could potentially interfere with reconsolidation of the OXY-CPP memory. At minimum, could the authors add information to the CPP Methods regarding the timing of the PSI/veh injections relative to removal from the CPP apparatus on day 5? If it was within 2 hours, this could disrupt reconsolidation. Was it a range of hours? At best, some Discussion of this seems to me to be highly relevant in the main text, unless I missed it.

Additional details related to the CPP protocol are included in the *Methods* section.

For OXY-CPP:

Upon completion of testing all groups on day 5, mice were brought back to their home cages and given either a single administration of PSI (1 mg/kg, i.p.) or vehicle. This injection occurred 2-4 h after the completion of the CPP preference test.

For CPP with PSI alone:

Habituation occurred the day prior to conditioning, and the testing phase was conducted 24-h after the final administration of PSI (2 mg/kg, s.c.), or vehicle.

Reviewer #2 (Remarks to the Author):

The authors addressed my concerns in their revisions.

We thank the *Reviewer* for their comments and previous suggestions.